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Quantification of Different *Eubacterium* spp. in Human Fecal Samples with Species-Specific 16S rRNA-Targeted Oligonucleotide Probes

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Species-specific 16S rRNA-targeted, Cy3 (indocarbocyanine)-labeled oligonucleotide probes were designed and validated to quantify different *Eubacterium* species in human fecal samples. Probes were directed at *Eubacterium barkeri*, *E. biforme*, *E. contortum*, *E. cylindroides* (two probes), *E. dolichum*, *E. hadrum*, *E. lentum*, *E. limosum*, *E. moniliforme*, and *E. ventriosum*. The specificity of the probes was tested with the type strains and a range of common intestinal bacteria. With one exception, none of the probes showed cross-hybridization under stringent conditions. The species-specific probes were applied to fecal samples obtained from 12 healthy volunteers. *E. biforme*, *E. cylindroides*, *E. hadrum*, *E. lentum*, and *E. ventriosum* could be determined. All other *Eubacterium* species for which probes had been designed were under the detection limit of 10^7 cells g (dry weight) of feces^{-1}. The cell counts obtained are essentially in accordance with the literature data, which are based on colony counts. This shows that whole-cell in situ hybridization with species-specific probes is a valuable tool for the enumeration of *Eubacterium* species in feces.

The genus *Eubacterium* (41) contains anaerobic, non-spore-forming, gram-positive rods which are distinguished from other genera mainly on the basis of negative metabolic characteristics (37). In the human intestinal tract, *Eubacterium* is the second most common genus after the genus *Bacteroides* and is more common than the genus *Bifidobacterium* (16). The importance of members of the genus *Eubacterium* has been reported previously (10, 24, 38, 48). Since the identification of *Eubacterium* species based on phenotypic traits requires experience and is time-consuming (5, 15, 16, 36, 44), many studies involving human fecal flora composition have refrained from looking at this genus (13, 20, 34, 42).

Considerable effort has been invested in the application of molecular techniques such as PCR (19, 27, 50) and hybridization (12, 25, 52) for the identification of fecal bacteria. However, the extraction, purification, and amplification of nucleic acids by PCR from fecal samples are often selective and limited. In contrast, whole-cell hybridization with fluorescently labeled, 16S rRNA-targeted oligonucleotide probes allows the determination of the numerical abundance of bacteria, including unculturable strains (39), in various ecosystems such as water (1, 26, 32), sludge (23), and fecal samples (17, 18, 29).

In contrast to the genus *Bifidobacterium*, for example, which is a phylogenetically and phenotypically well-defined taxon, *Eubacterium* species (9, 31) are phylogenetically diverse and thus it is not possible to design a genus-specific probe for *Eubacterium* spp. Therefore, probes for phylogenetic clusters or species have to be considered. The development and validation of a probe for the detection of a *Eubacterium* species has been reported recently (45).

The purpose of this study was to develop and apply 16S rRNA-targeted oligonucleotide probes to human feces for the detection of numerically dominant fecal *Eubacterium* species by whole-cell hybridization. The choice of bacteria was based on the reports by Finegold et al. (16) and Moore and Holdeman (36) and included *Eubacterium barkeri*, *E. biforme*, *E. cylindroides*, *E. contortum*, *E. dolichum*, *E. hadrum*, *E. lentum*, *E. limosum*, *E. moniliforme*, and *E. ventriosum*. The specific detection and enumeration of *Eubacterium* species with specific oligonucleotide probes facilitates the exploration of the microbial diversity in the human gut ecosystem and is expected to contribute to resolving the ecological role of bacteria in their environment.

MATERIALS AND METHODS

Organisms and culture conditions. All reference strains used in this study were obtained from the sources indicated in the appendix (Deutsche Sammlung von Mikroorganismen und Zellkulturen [DSMZ], Braunschweig, Germany; Deutsches Institut für Ernährungsforschung [DIFE], Bergholz-Rehbrücke, Germany; American Type Culture Collection [ATCC], Rockville, Md.). All DIFE strains are human fecal isolates from our laboratory and were identified by using the Vitek System (bioMérieux, Nürtingen, Germany). All strains, except for *Clostridium populeti* and *Eubacterium lentum*, which were grown on the media described in the DSMZ catalog, were cultured at 37°C under strictly anoxic conditions with N_2-CO_2 (80:20 [vol/vol]) as a gas phase (8, 22) in ST medium (ST), which contained (per liter): 9 g of tryptically digested peptone from meat, 1 g of proteose peptone, 3 g of meat extract, 4 g of yeast extract, 6 g of glucose, 3 g of NaCl, 2 g of Na_2HPO_4, 0.5 ml of Tween 80, 0.25 g of L-cystine, 0.25 g of L-cysteine · HCl, 0.1 g of $MgSO_4 \cdot 7H_2O$, 5 mg of $FeSO_4 \cdot 7H_2O$, and 3.4 mg of $MnSO_4 \cdot 2H_2O$ (pH 7.0).

Oligonucleotide probes. Oligonucleotides targeting the small subunit rRNA sequences of the *Eubacterium* spp. were designed with the Arb software package (46). The probe sequences were then checked by using the Check-Probe function of the Ribosomal Database Project (RDP) software package (31) and the alignment function of the EMBL database. The designed probes were named according to the nomenclature suggested by the Oligonucleotide Probe Database (OPD) (2). The theoretical dissociation temperature (T_D) of the designed probes was >50°C (47), and the G+C content was at least 50%. For practical use within this manuscript the probes were named in such a way that they indicate only the species name and the *Escherichia coli* (7) position, e.g., the probe for *E. barkeri* (S-S-E.bar-1237-a-A-18) was abbreviated E.bar1237.

Nucleic acid extraction. For nucleic acid extraction the bacterial strains were grown anoxically for 12 to 36 h in 100 ml of ST. The cells were harvested by centrifugation for 15 min at 4,000 × *g* at 4°C. The resulting sediment was subjected to nucleic acid extraction by following protocol number five of the InViTek DNA-Isolation Kit III (InViTek GmbH, Berlin, Germany) with a slight

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modification. To improve lysis, cells were first incubated for 30 min in 25% sucrose (wt/vol) in H_2O, centrifuged as described above, and diluted in 2 ml of Lysis-Buffer D (InViTek). The nucleic acid concentration was measured photometrically at 280 nm, and the quality was checked in ethidium bromide-stained agarose gels (1% [wt/vol]).

DNA amplification with PCR and specificity testing. The species-specific probes were used in PCR as reverse primers; the nucleotide sequence of the forward primer was 5'-AGAGTTTGATCMTGGCTCAG-3' (28). PCR amplification was performed with a PCR thermal cycler (Hybaid, Heidelberg, Germany) under the following conditions: denaturation at 94°C for 4 min, followed by 33 cycles of denaturation at 94°C for 1.5 min, annealing at 68°C for 1.5 min, and extension at 72°C for 1.5 min. After completion, an additional extension step was performed at 72°C for 6 min, and the samples were then chilled to 4°C. Reaction mixtures (final volume, 50 μl) contained 16 mM $(NH_4)_2SO_4$, 50 mM Tris-HCl (pH 8.8), 0.1% Tween 20 (vol/vol), 5 mM $MgCl_2$, 250 μM concentrations of each deoxynucleotide triphosphate, 50 pM concentrations of each oligonucleotide primer, 0.1% gelatin (wt/vol), and 2.5 U of *Taq* polymerase (InViTek). Template DNA was added to a final concentration of 1 ng μl^{-1}. The PCR products were visualized as described for the nucleic acid extraction. Probes were tested in PCR experiments with the reference organisms given in the appendix.

Detection limits of PCR-based identification in pure culture and feces. To determine the detection limit of PCR-based identification in pure cultures, defined amounts of cells from the late logarithmic growth phase were serially diluted in phosphate-buffered saline (PBS) (130 mM NaCl, 10 mM NaH_2PO_4-Na_2HPO_4; pH 7.2) and nucleic acids were extracted according to the above-described protocol. To determine the detection limit of this PCR-based method in feces, cells were diluted as described above and added to 100 mg of autoclaved feces and the nucleic acids were extracted.

Cell fixation for whole-cell hybridization. More than 90 reference strains representing the human intestinal flora were used for probe validation (see Appendix). As a positive control, the bacterium-specific probe S-D-Bact-0338-a-A-18 was used (3). Bacterial cells were grown overnight on Columbia blood agar plates (bioMérieux) incubated at 37°C in anaerobic jars under strictly anoxic conditions with N_2-CO_2 (80:20 [vol/vol]) as the gas phase. Bacteria that did not grow on these plates were cultured in 10 ml of ST before cell fixation and were subsequently harvested as described above. The fixation of the bacteria was dependent on their Gram stain reaction: gram-positive reference strains were diluted in PBS and adjusted to 10^9 cells/ml, centrifuged for 3 min at 2,000 × *g*, washed with 1 ml of PBS, and subsequently resuspended in 1 ml of 50% ethanol-PBS (vol/vol) (43), whereas gram-negative strains were resuspended in 1 ml of 4% paraformaldehyde (wt/vol) and stored for at least 4 h at 4°C (3). The cells were then sedimented by centrifugation and fixed by the same procedure as described for the gram-positive reference strains. The fixed cells were stored at −20°C until used.

Fecal samples. Fresh feces were collected from healthy humans without an antibiotic therapy in the last 6 months before the start of the study. For the analysis by whole-cell hybridization a 0.1-g (wet weight) feces specimen was added to 0.9 ml of sterile PBS and then mixed by inverting and vortexing the tube for 5 to 10 min. For total cell counts, 500 μl of this dilution was fixed with paraformaldehyde and ethanol, respectively, as described above for the pure cultures.

Hybridization. Hybridization was done on 70% ethanol rinsed and dried Teflon-coated slides (Marienfeld, Bad Mergentheim, Germany) with eight wells for independent positioning of the samples. Slides were silanized with 2% APES (3-aminopropyl-triethoxysilane) to improve cell distribution (30). Aliquots of fixed cells and fecal samples were spotted on single wells, air dried, and dehydrated by passing them through an ethanol series (60, 80, and 96% [vol/vol]) for 3 min each. Hybridizations were performed after addition of 2 μl (50 pmol/μl) of the species-specific Cy3 (indocarbocyanine; Interactiva, Ulm, Germany)-labeled probe in humid chambers overnight at the given temperature (Table 1). The hybridization buffer (10 μl) contained 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2), 0.01% (wt/vol) sodium dodecyl sulfate, and no formamide if not stated otherwise.

Probe specificity testing. To test the specificity of the designed probes the bacterial reference strains listed in the appendix were hybridized with the respective probe. Four strains per slide were tested, i.e., two wells per strain. One of the wells was used for the species-specific probe, and the other was used for the S-D-Bact-0338-a-A-18 probe (3), which detects all bacterial species. The optimal hybridization temperature (T_H) was determined experimentally for each probe. T_H is defined as the temperature at which a given probe hybridized exclusively with the target organism while S-D-Bact-0338-a-A-18 hybridized with all species given in the appendix. The slides were subsequently treated with the SlowFade Antifade Kit according to the instructions of the manufacturer (Molecular Probes, Leiden, The Netherlands). In addition, the hybridization temperature (T_E) of the designed probes was determined by the method of De Los Reyes et al. (11).

Analysis of fecal samples. Prior to counting, fixed fecal samples were briefly vortexed and subsequently centrifuged at 9 × *g* for 3 min. The supernatant of each sample was diluted in such a way that the numbers of fluorescing cells in a microscopic field (see below) were between 10 and 30. Of each dilution, 10 μl was applied to a separate well on the slide and treated as described above for pure cultures. To improve the permeabilization of the cell envelope, samples were treated with 10 μl of lysozyme buffer (100 mM Tris-HCl [pH 8.0], 50 mM EDTA, 1 mg of lysozyme/ml [130,000 U/mg; Boehringer, Mannheim, Germany]). Slides were incubated on ice for 7 min and subsequently dehydrated and hybridized as described above.

Microscopy and documentation. Total cell counts were determined with a Zeiss Axioplan 2 microscope (Zeiss, Jena, Germany) equipped with a 12-bit video camera (SensiCam; PCO, Kelheim, Germany) and several filters for epifluorescence microscopy. Enumeration was done by automated counting by using the KS400 3.0 software (Zeiss). The species-specific enumeration of cells was done manually with an Optiphot-2 microscope (Nikon, Düsseldorf, Germany) equipped with a G-2A filterblock for epifluorescence microscopy and a photomicrographic camera. Each well (6 mm in diameter) of the slides was subdivided into 25 different fields. Four wells were analyzed per sample (100 fields). The cell concentration (C) was calculated as follows: $C\ (ml^{-1}) = N_m \times DF_F \times M_F \times DF_S$, where N_m is the mean value of the cell numbers determined in 100 microscopic fields (four wells). DF_F (2,000 ml^{-1}) is the constant dilution factor resulting from the fixation process. M_F is the microscopic factor, which is defined as the total area per well divided by the area of the 25 fields inspected per well. The total area per well was 28.27 mm^2, whereas the area of the 25 fields was dependent on the microscope used.

For the Zeiss Axioplan 2 microscope the area was 0.0056 mm^2, and for the Optiphot-2 microscope the area was 0.0151 mm^2. DF_S is the dilution factor for each separate sample, which was necessary to obtain the 10 to 30 bacteria per field.

RESULTS

Probe design. Eleven different oligonucleotide probes were designed based on comparative analysis by using the ARB program and checked with the RDP and the EMBL databases. Alignments of all chosen 16S rRNA sequences were screened for targets that enable species-specific discrimination. Table 1 shows the probe sequences; their target sites; their T_D, T_H, and T_E values (for definitions, see Materials and Methods); and alignments of the potential 16S rRNA binding sites of nontarget organisms. Probes were named according to the OPD (2). Since the RDP contains two different 16S rRNA sequences for *E. cylindroides*, we designed two different species-specific probes and used an equimolar mixture of both probes for the detection of *E. cylindroides*. The E.lim1433 probe designed for the detection of *E. limosum* does not allow its discrimination from *E. callanderi*, a species quite closely related to *E. limosum*. The differentiation between the two organisms is based on minor phenotypic differences (40, 51). Hence, E.lim1433 is a dispecies-specific probe.

E. lentum was recently reclassified as *Eggerthella lenta* gen. nov., comb. nov. (49). However, since this organism was originally described as a numerically dominant *Eubacterium* species (16), we included it in our study and refer to it here as *E. lentum*.

Specificity of the probes in whole-cell hybridization. Since the hybridization conditions used for fluorescence in situ hybridization (FISH) differ from those used for the determination of T_E (11, 45), hybridization experiments with reference strains originating from the human and animal gastrointestinal tract were done at T_H (Table 1). As a positive control, the bacterium-specific probe S-D-Bact-0338-a-A-18 (3) was used. In pure culture, all designed probes resulted in a hybridization signal of similar intensity, which worked equally well on the first try in pure cultures. The probes designed for the detection of *E. barkeri* (E.bar1237), *E. biforme* (E.bif462), *E. cylindroides* (E.cyl461 and E.cyl466), *E. dolichum* (E.dol183), *E. hadrum* (E.had580), *E. lentum* (E.len194), *E. limosum* (E.lim1433), *E. moniliforme* (E.mon84), and *E. ventriosum* (E.ven66) hybridized to the corresponding target organism but not to the other organisms listed in the appendix. E.con1122, which had been designed for *E. contortum*, cross-hybridized with the closely related *E. fissicatena*. An increase in stringency by elevation of the temperature or the formamide concentration did not prevent the unspecific binding of E.con1122 to *E. fissicatena*. We therefore decided to use E.con1122 for the detection of both organisms. E.con1122 is therefore a dispecies-specific probe. A

TABLE 1. Developed and validated 16S rRNA-targeted species-specific oligonucleotide probes for *Eubacterium* spp. and the corresponding sequence accession numbers[a]

Probe (accession no.; T_D, T_H, T_E [°C]) and related strains	Sequence
S-S-E.bar-1237-a-A-18 (M23927; 51, 51, 53)	3'TTACCCAACCCTGTTTCC'
	5'AAUGGGUUGGGACAAAGG' (target)
Acidaminococcus fermentans	.....UCG.CA.....
Ruminococcus productus	C.AU..C...U.....
S-S-E.bif-0462-a-A-18 (M59230; 51, 51, 56)	3'CCCTTACTACTCACTCAC'
	5'GGGAAUGAUGAGUGAGUG' (target)
Eubacterium cylindroides ATCC 27803	.....CC...G...
Streptococcus pleomorphus	.....UCG.....
Fusobacterium mortiferum	...UG....CU.C..N..
Fusobacterium varium	...UG....CU.C..N..
S-S-E.con-1122-a-A-20 (L34615; 61, 55, 55)	3'GGCCTAAACCGCTGGCTACT'
	5'AGUAGCCAGCGGUUUAGGCC' (target)
Eubacterium fissicatena	.....G.C.G.
S-St-E.cyl-0461-a-A-18 (L34617; 53, 51, 53)	3'TCCCTTACTAGGCACCCA'
	5'AGGGAAUGAUCCGUGGGU' (target)
Eubacterium biforme	.....GA...A..
Eubacterium uniforme	G.N..G.A.NN.....
Eubacterium cylindroides ATCC 27528	...A....C.AU.....
Fusobacterium varium	GA..G...U....A....
S-St-E.cyl-0466-a-A-18 (L34616; 53, 51, 53)	3'TACGATACACCCACTGCC'
	5'AUGCUAUGUGGGUGACGG' (target)
Clostridium innocuum	.....G.A.....
Eubacterium cylindroides ATCC 27803	..A.CC.....U..
Clostridium spiroforme	..A...CC.A.....
Eubacterium biforme	..A..GA...A....U..
S-S-E.dol-0183-a-A-18 (L34682; 55, 51, 53)	3'TGTCTCCGAGATGCCTCG'
	5'CGAGGCAUCUCGGAGACA' (target)
S-S-E.had-0579-a-A-20 (ARB 671C; 59, 54, 54)	3'GACTTGCCATACCACCTACG'
	5'CGUAGGUGGUAUGGCAAGUC' (target)
Eubacterium ventriosum	.....C..C.....
Eubacterium uniforme	.....C.A.....
Bacteroides fragilis	.....AC...U.....
S-S-E.len-0194-a-A-18 (AB011817; 55, 51, 53)	3'CCTTGCCGTCTGGGCTTT'
	5'AAAGCCCAGACGGCAAGG' (target)
Eubacterium ventriosum	.....C..C.....
Eubacterium uniforme	.....C.A.....
Bacteroides fragilis	.....AC...U...
S-S-E.lim-1433-a-A-18 (M59120; 55, 51, 59)	3'TCGGACACTCTCTTGGCG'
	5'AGCCUGUGAGAGAACCGC' (target)
Eubacterium barkeri	....A.....C.....
Pseudoramibacter alactolyticus	...A.....C.....
Eubacterium cylindroides ATCC 27528	....G...GC.U.....
Eubacterium tortuosum	....G...GC.U.....
S-S-E.mon-0084-a-A-18 (L34622; 53, 51, 52)	3'CCGCTAATCCATTTCCCG'
	5'CGGGAAAUGGAUUAGCGG' (target)
S-S-E.ven-0066-a-A-18 (L34421; 51, 55, 50)	3'TCTGTCCAAGGTGCTTCG'
	5'CGAAGCACCUUGGACAGA' (target)

[a] Alignments of the probe sequences and their 16S rRNA target sites. Also shown are the potential 16S rRNA binding sites of nontarget organisms commonly found in human and animal feces. Accession numbers are from GenBank except for *E. hadrum* which only has an ARB accession number (46). Probes are named in accordance with the OPD (2). Sequences show the closest nontarget sequences available in the databases. Only nucleotides that differ from the target sequences are shown. N indicates a not yet determined nucleotide. The first T_D refers to the theoretical dissociation temperature based on the contribution of GC and AT pairs to the oligonucleotide duplex stability (45). T_H refers to the hybridization temperature used in the study. T_E is related to the hybridization temperature as determined by the method by De Los Reyes et al. (11).

probe for *E. rectale* (S-S-E.rec-0574-a-A-18) was designed, and its specifity was tested in FISH experiments. Since this probe cross-hybridized with nontarget organisms, E.rec574 was not included in the probe panel. An increase in stringency by elevation of the temperature or the formamide concentration did not prevent unspecific binding. Examples of whole-cell hybridization with E.bar1237 in pure culture and E.bif462 in fecal samples are given in Fig. 1.

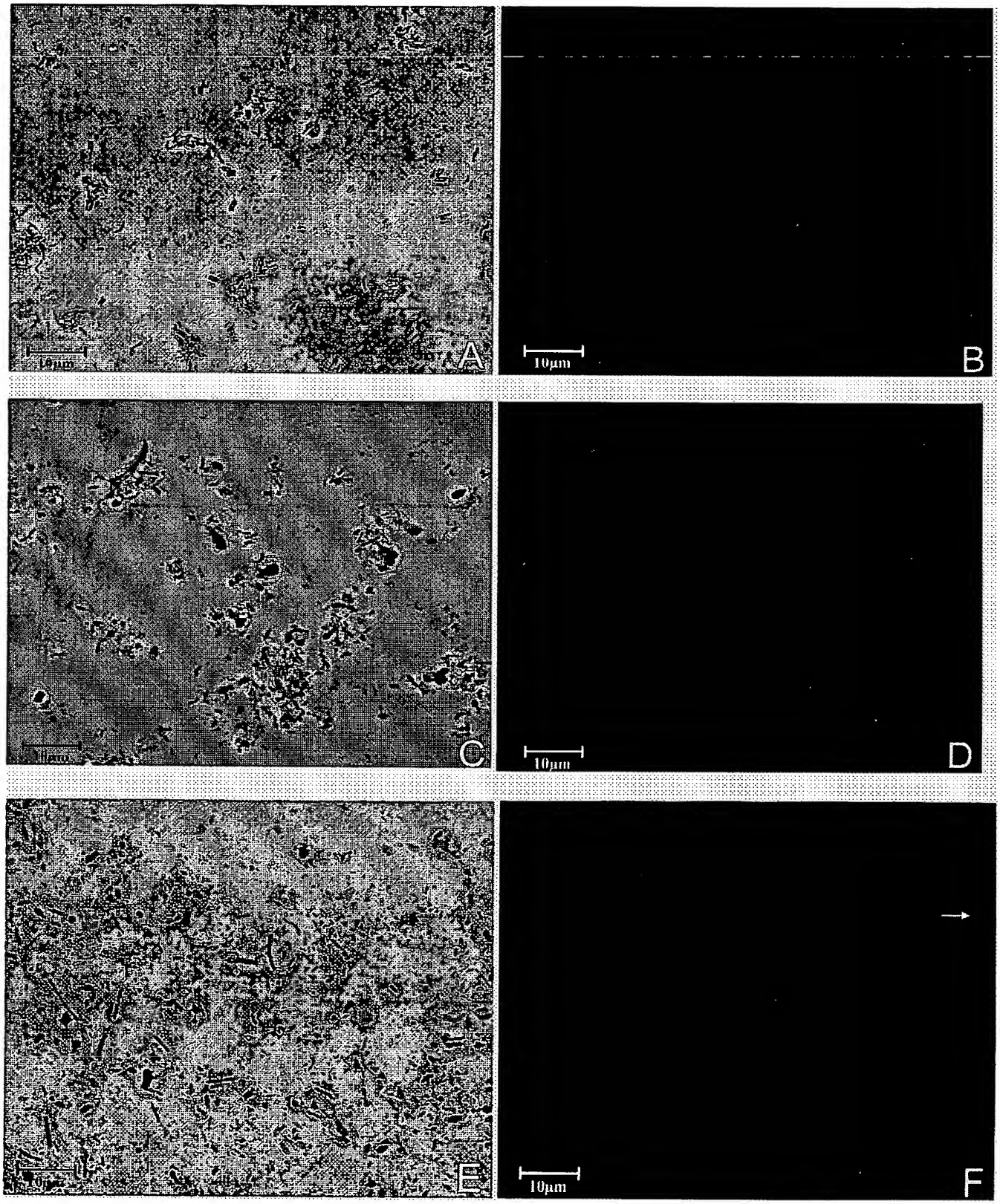

FIG. 1. Phase-contrast and epifluorescence micrographs of pure culture and fecal samples. (A and B) Pure culture of *E. barkeri* hybridized with Cy3-labeled E.bar1237 (S-S-E.bar-1237-a-A-18) probe. Panels A and B show the same microscopic field viewed by phase-contrast and epifluorescence microscopy, respectively. (C and D) Detection of *E. biforme* in a fecal sample with E.bif462 (S-S-E.bif-0462-a-A-18). Panels C and D show the same microscopic field viewed by phase-contrast and epifluorescence microscopy, respectively. (E and F) Detection of *E. hadrum* in a fecal sample with E.had579 (S-S-E.had-0579-a-A-20) and cross-hybridization to an unknown coccoid-shaped bacterium. Panels E and F show the same microscopic field viewed by phase-contrast and epifluorescence microscopy, respectively. In panel E the unknown coccoid-shaped bacterium is marked with an arrow. Both visible bacteria (coccoid shaped and rod shaped) were detected with E.had579.

To determine the applicability of the validated probes to fecal samples, each probe was used to enumerate target bacteria in fecal samples from 12 healthy volunteers. Cell counts, relative proportions, and incidences of the target organisms in the 12 subjects are summarized in Table 2. The detection limit was 10^7 cells g (dry weight)$^{-1}$.

With E.had579, two types of organisms were detected in fecal samples, a rod-shaped organism and a coccoid organism. The latter was obviously not contained in the panel of reference organisms used for testing the probe specificity. Since the two types of organisms could be distinguished easily by their morphology, only the rod-shaped organisms detected with E.had579 were considered to be *E. hadrum*.

E. hadrum was detected in 11 of 12 subjects at 8×10^7 to 5.59×10^8 cells g (dry weight)$^{-1}$. The probes E.bar1237, E.bif462, E.len194, E.cyl461, E.cyl466, E.con1123, and E.ven113 detected only bacteria with the expected morphology and were therefore considered to be species specific. The probes E.dol183, E.lim1433, and E.mon84 gave no signals in fecal samples. Results are presented in Table 2.

Use of *Eubacterium* probes in PCR experiments. In order to increase the sensitivity of detection, 6 of the 11 probes were used in PCR experiments as primers. All primer sets resulted in bands of the expected size as follows: *E. barkeri*, 1,229 bp; *E. biforme*, 479 bp; *E. cylindroides* ATCC 27803, 480 bp; *E. cylindroides* ATCC 27528, 487 bp; *E. limosum*, 1,424 bp; and *E. ventriosum*, 62 bp. No amplification product could be detected with other organisms listed in the appendix.

The sensitivity of the PCR methodology was first determined in pure cultures and subsequently for *E. limosum* in autoclaved feces (Fig. 2). In pure cultures, *E. barkeri*, *E. biforme*, and *E. limosum* could be detected at concentrations of 10^2 cells ml^{-1}, and *E. cylindroides* and *E. ventriosum* could be detected at concentrations of 10^3 cells ml^{-1} (data not shown). The detection limit for *E. limosum* in autoclaved feces was by several orders of magnitude lower than in pure culture. It varied between 10^5 and 10^7 cells g (dry weight)$^{-1}$.

DISCUSSION

Eleven different probes were designed and applied both in whole-cell hybridization and PCR to detect various species of the genus *Eubacterium* in human feces. Several studies reported on the development of specific probes for various genera, including *Bifidobacterium* (29), *Streptococcus*, and *Lactococcus* or members of the *Bacteroides fragilis*, *Clostridium histolyticum*, *Clostridium lituseburense*, and *Clostridium coccoides-Eubacterium rectale* groups (17), respectively. Species-specific probes have been developed for a few species only (6, 14, 17, 45). The application of the newly designed probes to fecal samples allowed us to enumerate a number of *Eubacterium* species previously reported to be present in the human intestinal tract (15, 16, 21, 36). The cell counts obtained in our study for *E. biforme*, *E. contortum*, *E. cylindroides*, *E. lentum*, and *E. ventriosum* are essentially in agreement with the published data, which were obtained with classical microbiological enumeration techniques (Table 2) (15, 16, 21, 36). *E. hadrum* was only reported in one study (21). None of the other probes developed (E.bar1237, E.dol183, E.lim1433, and E.mon84) gave a signal in feces from any of the 12 subjects. The only exception was the probe for *E. barkeri*. However, with this probe the cell numbers detected in feces were too low to get reliable counts. Our experiments indicated that the detection limit for a given target organism in feces was approximately 10^7 cells g (dry weight)$^{-1}$. Considering the high total cell counts in feces of about 10^{11} to 10^{12} cells g (dry weight)$^{-1}$ (29, 45; and

TABLE 2. Quantification of *Eubacterium* species in human feces with whole-cell hybridization compared to literature data

Eubacterium species	Designed probe	Range in subjects testing positive (mean)	Subjects testing positive (n = 12)	Range (%) of total fecal flora in subjects testing positive	Data from Finegold et al. (15)[a]		Data from Holdeman et al. (21)	
					Range in subjects testing positive (mean)	% Persons testing positive (n = 141)	Mean count[a]	% Total flora (n = 3)
E. barkeri	E.bar1237	ND[b]	0	ND	9.1–10.5 (9.8)	3.5	ND	ND
E. biforme	E.bif462	7.59–9.1 (8.35)	6	0.08–3.3	8.3–11.3 (9.7)	8	9.48	1.2
E. contortum	E.con1122	9.2	1	0.8	4.2–12.7 (9.5)	26.2	ND	ND
E. cylindroides	E.cyl461, E.cyl466	7.7–8.24 (7.97)	2	0.03–0.07	3.5–11.9 (9.6)	23	ND	ND
E. dolichum	E.dol183	ND	0	ND	6.5–11.0 (8.6)	2.8	ND	ND
E. hadrum	E.had579	7.9–8.7 (8.3)	11	0.04–0.18	ND	ND	8.77	0.23
E. lentum	E.len194	8.1–8.5 (8.29)	4	0.1–0.6	3.6–11.6 (9.3)	42.6	ND	ND
E. limosum	E.lim1433	ND	0	ND	5.8–10.8 (8.7)	5.7	8.63	0.17
E. moniliforme	E.mon84	ND	0	ND	8.0–11.1 (9.9)	6.4	ND	ND
E. ventriosum	E.ven66	7.5–9.3 (8.4)	2	0.03–1.7	4.0–12.3 (9.2)	12.1	9.1	0.5

[a] Ranges and mean counts of bacteria are expressed as the number of organisms ($\log_{10}$) per gram (dry weight) of feces.
[b] ND, not detected.

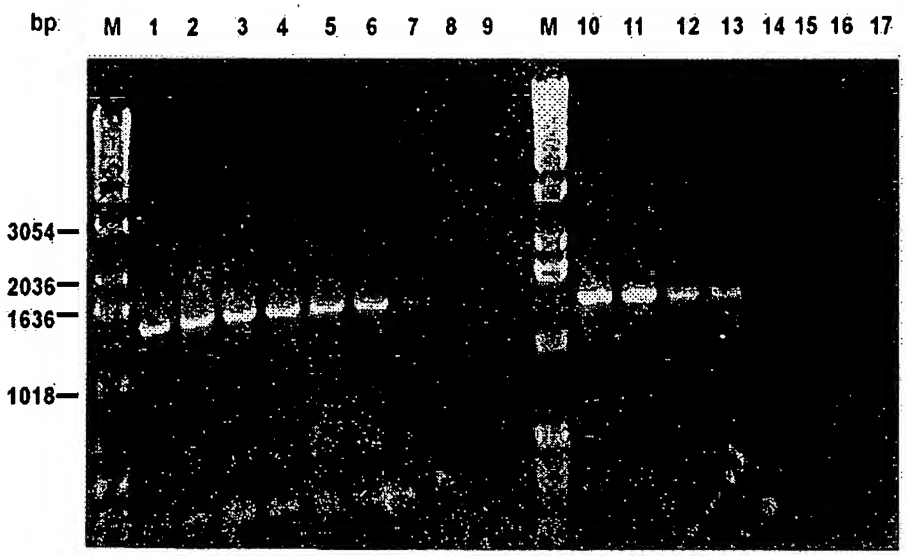

FIG. 2. PCR products obtained with genomic DNA of *E. limosum* from different cell numbers in autoclaved feces and pure culture on 1% agarose gel. Lanes M are the molecular marker (1-kb ladder). Lanes 1 to 9 show the PCR products obtained with cell numbers in the range from 10^8 to 10^0 from pure culture. Lanes 10 to 17 show PCR products obtained with cell numbers in the range from 10^6 to 10^1 which were added to autoclaved feces.

this study), this is not so bad as it may appear, since it is equivalent to identifying 1 cell in 100,000.

To overcome this detection limit, PCR-based amplification of 16S rDNA was used as an alternative method. The development of PCR methods for the detection of *E. biforme* and *E. limosum* with species-specific primers in fecal samples was reported earlier (50). Of the two organisms, only *E. limosum* was found in human fecal samples. Our species-specific primer sets for the detection of *E. barkeri*, *E. biforme*, *E. cylindroides*, *E. limosum*, and *E. ventriosum* identified the presence of these organisms at concentrations of 10^2 to 10^3 cells ml^{-1} in pure culture but not in autoclaved feces to which the target cells had been added from pure cultures. The detection limit increased to 10^5 to 10^7 cells g (dry weight)$^{-1}$. The increase in the detection limit is due to fecal substances that inhibit PCR. These inhibitors have been identified as complex polysaccharides (35). None of the methods we have used for nucleic acid extraction (19, 33, 50) from feces was successful in removing these PCR inhibitors. Therefore, we decided to use the developed probes only for whole-cell hybridization.

One important difference between previous studies (16) and the present study lies in the number of subjects analyzed. Our study included 12 subjects, whereas in the Finegold et al. study (16) 141 subjects were analyzed. It is important to note that Finegold and coworkers (16) detected some *Eubacterium* species, e.g., *E. barkeri*, *E. dolichum*, *E. limosum*, and *E. moniliforme*, in high numbers but only in a few subjects. The inability to detect these species in our study may therefore be due to the fact that only a relatively limited number of subjects was examined.

In spite of the limitations of our study, the molecular approach used here avoids a number of problems associated with the phenotypic detection of *Eubacterium* species. Since most *Eubacterium* species are fastidious in their nutritional requirements, no special enrichment medium is available (37). Furthermore, the genus *Eubacterium* is phylogenetically heterogeneous and some of the *Eubacterium* species are pleomorphic, e.g., *E. biforme*, *E. cylindroides*, or *E. dolichum*, depending on the culture method. Hence, identification and determination at the species level is not a trivial task.

The advantage of whole-cell hybridization for the detection of bacteria in contrast to other molecular methods such as PCR (19, 27, 50) and dot blot hybridization (12, 25, 52), lies in the fact that the former method also allows the distinction of bacteria based on their morphology, as cells can be visualized in the microscope.

Since the probes are designed on the basis of the currently available 16S rRNA sequences (31, 46), the nonspecific detection of bacteria not included in the list of reference organisms but present in the habitat cannot be excluded. However, even then the targeted organism may still be identified based on its morphology. We were thus able to distinguish *E. hadrum* from a coccoid bacterium, which was obviously not among the 90 reference strains but was present in human feces.

The application of whole-cell hybridization to the fecal samples also has its limitations, the major one being the lack of sensitivity. This may be partly due to the fact that the number of rRNA target molecules in cells occurring in natural habitats is lower than in pure cultures because nutritional limitation and other competitive factors influence the cellular ribosome content (4, 29). It is therefore not surprising that the fluorescence signal of cells in fecal samples is lower than in pure culture (29). In addition, nonspecific fluorescence may hamper the visualization of bacteria. To increase the sensitivity of whole-cell hybridization, we used probes labeled with Cy3, whose signal strength is superior to that of other fluorescent molecules (1). In addition, fecal samples were treated with lysozyme to improve cell permeabilization, and the SlowFade Antifade Kit was used to reduce fading during microscopy.

In conclusion, whole-cell hybridization with species-specific oligonucleotide probes can assist in fast and accurate analysis of the *Eubacterium* spp. in fecal samples, provided the concentration of the target organisms is above the detection limit of 10^7 cells g (dry weight)$^{-1}$. The oligonucleotide probes developed in this study will help to refine our present knowledge of this important group of bacteria in the intestinal tract.

APPENDIX

The strains considered in this study included *Acidaminococcus fermentans* (DSMZ 2073), *Actinobaculum suis* (DSMZ 20639), *Bacte-*

roides distasonis (DSMZ 20701), *Bacteroides ovatus* (DSMZ 1896), *Bacteroides* sp. (*B. fragilis*, DIFE), *Bacteroides* sp. (*B. vulgatus*, DIFE),* *Bacteroides thetaiotaomicron* (DSMZ 2079), *Bifidobacterium adolescentis* (ATCC 15703), *Bifidobacterium angulatum* (ATCC 27535), *Bifidobacterium animalis* (ATCC 25527), *Bifidobacterium bifidum* (ATCC 29521), *Bifidobacterium breve* (ATCC 15700),* *Bifidobacterium catenulatum* (ATCC 27539), *Bifidobacterium dentium* (ATCC 27534), *Bifidobacterium infantis* (ATCC 15697), *Bifidobacterium infantis* (ATCC 15702),* *Bifidobacterium infantis* (ATCC 25962), *Bifidobacterium longum* (ATCC 15707), *Bifidobacterium longum* (ATCC 15708), *Butyvibrio fibrisolvens* (DSMZ 3071),* *Clostridium acetobutylicum* (ATCC 824), *Clostridium barati* (DSMZ 601), *Clostridium bifermentans* (DSMZ 46282), *Clostridium butyricum* (DSMZ 10702), *Clostridium cellobioparum* (DSMZ 1351), *Clostridium clostridiforme* (DSMZ 933), *Clostridium coccoides* (DSMZ 935),* *Clostridium innocuum* (DSMZ 1286), *Clostridium pasteurianum* (DSMZ 525), *Clostridium perfringens* (DSMZ 756), *Clostridium populeti* (DSMZ 5832), *Clostridium propionicum* (DSMZ 1682), *Clostridium saccharolyticum* (DSMZ 2544), *Clostridium sartagoformum* (DSMZ 1292), *Clostridium sordellii* (DSMZ 2141), *Clostridium* sp. (DSMZ 523), *Clostridium* sp. (*C. butyricum*, DIFE), *Clostridium* sp. (*C. perfringens*, DIFE), *Clostridium spiroforme* (DSMZ 1552),* *Clostridium sporosphaeroides* (DSMZ 1294), *Clostridium tyrobutyricum* (DSMZ 663), *Clostridium xylanolyticum* (DSMZ 6555), *Coprococcus catus* (ATCC 27761), *Enterococcus hirae* (DSMZ 20160),* *Enterococcus* sp. (*E. cassiliflavus*, DIFE), *Enterococcus* sp. (*E. durans*, DIFE), *Enterococcus* sp. (*E. faecalis*, DIFE), *Enterococcus* sp. (*E. faecium*, DIFE), *Escherichia hermanii* (ATCC 33650), *Escherichia* sp. (*E. coli*, DIFE), *Eubacterium aerofaciens* (DSMZ 3979), *Eubacterium barkeri* (ATCC 25849), *Eubacterium biforme* (DSMZ 3989), *Eubacterium contortum* (DSMZ 3982), *Eubacterium cylindroides* (ATCC 27528), *Eubacterium cylindroides* (ATCC 27803), *Eubacterium dolichum* (DSMZ 3991), *Eubacterium eligens* (DSMZ 3376), *Eubacterium fissicatena* (DSMZ 3598), *Eubacterium hadrum* (DSMZ 3319),* *Eubacterium hallii* DSMZ 3353),* *Eubacterium lentum* (DSMZ 2243), *Eubacterium limosum* (DSMZ 20543), *Eubacterium moniliforme* (DSMZ 3984), *Eubacterium multiforme* (DSMZ 20694), *Eubacterium ramulus* (ATCC 29099), *Eubacterium rectale* (ATCC 33656), *Eubacterium saburreum* (DSMZ 3986), *Eubacterium siraeum* (DSMZ 3996), *Eubacterium* sp. (*E. hadrum*, DIFE), *Eubacterium* sp. (*E. ramulus*, DIFE), *Eubacterium* sp. nov. (DIFE),* *Eubacterium tenue* (DSMZ 20695), *Eubacterium tortuosum* (DSMZ 3987), *Eubacterium uniforme* (ATCC 35992), *Eubacterium ventriosum* (ATCC 27560), *Fusobacterium mortiferum* (ATCC 25557), *Fusobacterium naviforme* (DSMZ 20699), *Fusobacterium necrogenes* (ATCC 25556), *Fusobacterium nucleatum* subsp. *polymorphum* (DSMZ 20482), *Fusobacterium varium* (ATCC 8501), *Klebsiella* sp. (*K. pneumoniae*, DIFE), *Lactobacillus fermentum* (DSMZ 20052), *Lactobacillus gasseri* (DSMZ 20243), *Lactobacillus murinus* (DSMZ 20452), *Lactobacillus plantarum* (DSMZ 20174), *Lactobacillus reuteri* (DSMZ 20016), *Lactobacillus* sp. (*L. acidophilus*, DIFE), *Megasphaera* sp. (DIFE), *Peptostreptococcus anaerobius* (DSMZ 2949),* *Peptostreptococcus asaccharolyticus* (DSMZ 20463), *Peptostreptococcus prevotii* (DSMZ 20548),* *Pseudoramibacter alactolyticus* (DSMZ 3980), *Ruminococcus hansenii* (DSMZ 20583),* *Ruminococcus productus* (DSMZ 2950), *Streptococcus intermedius* (DSMZ 20573), *Streptococcus pleomorphus* (DSMZ 20574), *Veillonella parvula* (DSMZ 2008), and *Veillonella* sp. (DIFE). Strains marked with an asterisk were not used in PCR validation experiments.

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